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Original Article

Arginase Inhibitory, Antioxidant Activity, Total Phenolic Content and Total Flavonoid Content of Ethyl Acetate Extract of *Caesalpiniaturtuosa* Roxb Stem Bark

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ABSTRACT

Objective: The purpose of this study is to investigate arginase inhibition, antioxidant activity, total phenolic content and total flavonoid content of ethyl acetate extract of *Caesalpiniaturtuosa Roxb*. **Material and method:** stem bark of *Caesalpiniaturtuosa* Roxb was extracted using hexane, ethyl acetate and methanol subsequently. The ethyl acetate extract was fractioned. Then, the fractions were subjected to arginase inhibition, antioxidant activity, total phenolic content and total flavonoid assay. Correlation was considered by statistical analysis. **Result:** Out of eight fractions, two fractions have no activity. Two fractions (3 and 6) have strong activity in arginase with inhibition 90.72 % and 91.41% respectively. Fraction 3 and 6 have strong antioxidant activity with IC_{50} 25.98 µg/mL and 48.01 µg/mL respectively. Statistical analysis shows arginase inhibitor activity was not related with antioxidant activity, total phenolic content and total flavonoid content in this plant. **Conclusion:** Activity in arginase inhibition of fraction from ethyl acetate extract of *Caesalpiniaturtuosa* Roxb are not related to antioxidant, total phenolic and flavonoid content.

Key words: Arginase, Antioxidant, *Caesalpiniaturtuosa* Roxb, Flavonoid.

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INTRODUCTION

Endothelial dysfunction is a pathogenic factor of many diseases such as diabetes, arthritis, and hypercholesterolemia.[1-3] Endothelial cell dysfunction also contributes to cardiovascular disease development like atherosclerosis, hypertension, myocardial infarction, and stroke.[4-6] Major contributor for endothelial dysfunction is Nitric oxide (NO) bioavailability decreasing. Normally, NO is produced trough converting L-arginine catalyzed by NOS.[6,7] NO is a potent vasodilator and essential regulator for vascular tone and blood pressure.[8] NO also plays a role in regulating hemostasis, and leukocyte adhesion and smooth muscle cell proliferation.[6]

L-arginine ureahydrolase, known as arginase, is a manganese metalloenzyme in the hepatic urea cycle that converts l-arginine to ornithine and urea.[3,5,7] Arginase plays two crucial roles homeostatic purposes, riding of ammonia through urea synthesis and producing ornithine from L-arginine. Ornithine is the precursor for polyamines and prolines.[7] Increasing of arginase activity reduce the supply of L-arginine needed by nitric oxide (NO) synthase to produce NO. NO is a crucial vasoconstrictor.[9] Besides, excess of ornithine causes vascular structural problems and neural toxicity.[7] Also related to abnormal arginase activity, previous study showed that urea at certain concentrations can induce reactive oxygen species (ROS) production which further cause activation of pro-inflammatory pathways, and inactivation of the anti-atherosclerosis.[10]

Regulation of arginase activity is one mechanism suggested to cure several health problems that endothelial dysfunction related. Inhibiting arginase activity has proven to be beneficially useful in various illness such as hypertension, erectile dysfunction, diabetic renal injury, myocardial ischemia-reperfusion injury.[9]

Arginase inhibitor is compounds that interact with arginase which reduce the activity of this enzyme and increase the availability of L-arginine as substrate for NOS resulting in NO balancing. First generation of arginase inhibitor is N-hydroxy-l-arginine (NOHA) and N-hydroxy- nor-l-arginine (nor-NOHA), characterized by N-hydroxy- guanidinium side chains.[6] Nor NOHA is synthetized most potent inhibitor known.[9] The next generation of arginase inhibitor is S-(2-boronoethyl)-L-cysteine (BEC) and 2-(S)-amino-6-boronohexanoic acid (ABH). However, these three compounds have several problems in therapeutic use. Nor NOHA has very short life time, while BEC and ABH are constrained in pharmacokinetics profile and toxicity.[4,9,11] Therefore, discovery the more effective and safe arginase inhibitor is required.

Several aminoacid such as glysine, L-alanin, L-valin activity in inhibiting arginase. Chloroquineand some antiretroviral, like darunavir and atanazavirare known have high potency on inhibit arginase activity.[12]

Many plant extracts were found to inhibit arginase activity. Ethanol extract of some part from *Melastosomamalabathricum* was found to have arginase activity inhibition. The highest activity was found from leaves extract 81.26 %, followed by

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flower, fruit, and stem with inhibition activity of 73.39 %, 67.63 % and 61.61 % respectively.[1] The methanol extract of *Sterculiamacrophylla leaves* was found to have IC_{50} 114,659 µg/mL.[2]

Polyphenolic compound, such as cholorogenic acid and piceatannol exhibited inhibitory activities of arginase with IC_{50} 10.6 and 12.1 µg/mL, respectively. These two compounds were identified as competitive inhibitor for arginase.[13] Quercetin, kaempferol, epicatechin show inhibitory activity on bovine arginase with IC_{50} of 31.2, 179.1, and 19.9 µg/ml.[13] Fisetin has higher potency inhibiting arginase four and ten times greater than quercetin and luteolin, respectively.[14]

The bioactive compounds of glucoside flavonoids, isolated from *Cecropiapachystachya* and characterized as Orientin (luteolin-8-C-glucoside), show activity as arginase inhibitor with IC50 of15.9 µg/mL.[15] Orientin and isoorientin inhibit arginase activity through uncompetitive inhibition.[16] A new type of flavonoid and other sever known compound were isolated from methanol extract of *Scutellariaindica*. Two flavonoids characterized as *(2S)-5,7-dihydroxy-8,20-di-methoxyflavanone and (2S)-5,20,50-trihydroxy-7,8-dimethoxyflavanone* show inhibitory activity of arginase with IC_{50} of 25.1 and 11.6 µg/mL[3].

Several flavonoids, diterpenes, and steroids, have been isolated from genus Caesalpinia.[17] Various pharmacological studies conducted on several Caesalpinia species showed activity in inhibiting arginase activity. Arginase inhibitory activity test conducted for the bark extract of *Caesalpiniacoriaria (Jacq.)* showed that the ethyl acetate and methanol extracts have average inhibition values of 14.43 and 33.59%, respectively.[18] Methanolic extract of *C. pulcherrima (L.)* Sw. stem bark showed activity in inhibiting arginase 21.969 µg/mL.[4] Methanolic extract of *Caesalpiniaferrea* C. Mart showed arginase inhibitory activity of 12.81%.[19]

The purpose of this study is to investigate arginase inhibition of eight fractions from Ethyl acetate extract of *Caesalpiniaturtuosa Roxb*. Antioxidant activity, total phenolic content and total flavonoid content were determined to active fraction in order to predict the variables that related to activity of arginase inhibition.

MATERIAL AND METHODS

Material

Caesalpiniaturtuosa Roxb was collected in July 2018 from Bogor Botanical Garden, Bogor, West Java, Indonesia. n-hexane, ethyl acetate, and methanol were purchased from local suppliers. Silica gel 60 (Merck, Germany), aqua pro injection, arginase enzymes (Sigma, Singapore), nor-NOHA standard (Cayman, USA), L-arginine (Sigma, Singapore), maleic acid (Sigma, Singapore), manganese sulfate (Sigma, Singapore), urea assay kits (Quan- tichrom® Bioassay, United States), dimethyl sulfoxide (Merck, Germany), methanol pro analysis (Merck, Germany), ethanol pro analysis (Merck, Germany), foline-ciocalteu (Merck, Germany),

Extraction

Dried powdered stem bark *C.turtuosa* Roxb was extracted using multistage maceration with n-hexane, ethyl acetate, and methanol subsequently. From these step, obtained three crude extract. Ethyl acetate extract then was evaporated and fractionated by using classic column chromatography using gradient eluent. N-hexane, ethyl acetate and methanol were used respectively.

Arginase inhibition assay

The inhibition of arginase activity assay was performed by following the enzyme protocol from Sigma Aldrich and urea assay kit obtained from Abnova Corporation, Taiwan (KA 1652) with slight modification. Nor-NOHA acetate was used as the standard. Briefly, 10 µL of the 100 µg/ml extract solution was added into the well followed by 15 µL of arginase solution (1 U/mL) and 20 µL L-arginine 570 mM as the substrate of arginase, consecutively. Dimethyl sulfoxide was used to replace sample/standard in control wells. The mixture was shaken then incubated for 30 min at 37˚C.100 µL of urea assay kit (kit A: kit B = 1 : 1) was added into each well to stop the reaction, then incubation for 60 min at room temperature. The absorbance was measured at 430 nm using micro-plate reader (Versamax micro-plate reader, USA).

Antioxidant activity

Antioxidant activity was determined using DPPH scavenging ability assay, conducted in a 96-well plate according to previously used method Zhang lu et al. Briefly, 20 µL of samples in different concentrations (2.5-80 mg/L) and 180 µLof 0.114 mM DPPH in methanol were added to each well. After 40 minutes of incubation in the dark absorbance, it was read at 517 nm using micro-plate reader. The scavenging ability (%) was calculated as follows:

Scavenging ability (%) = [(B-S)/B]×100%

B was the absorbance of control without sample, S was the absorbance of sample. All tests were performed in triplicate. Concentration of sample resulting 50% inhibition on DPPH was calculated.

Determination of total phenolic content

Total phenolic content was measured using the 96-well microplate with Folin–Ciocalteu reagent, adapted from Farasat et al with minor modification.[20] 20 µL of the sample/standard, 100 µL of reagent Folin–Ciocalteu (1:10) were added to each well, shaken for 60 s then allowed to last for 4 min. 80 µL of sodium carbonate solution (100 g/L) were added into the well forward, shaken for 1 minute. The absorbance was measured at 750 nm using the micro plate reader (Versamax Microplate Reader) after incubated for 2 hours at room temperature. The calibration curve for determining the phenolic contents was prepared by using gallic acid (6.25–200 mg/L) as positive control.

Determination of total flavonoid content

Flavonoid content was determined by following method adapted from Farasat et al using the 96-well microplate with slight modification.[20] Briefly, 20 µL of each sample/standards were added to well followed with 20 µL of 10 % aluminum chloride, 20 µL of 1 M sodium acetate, and 180 µL of distilled water, consecutively. The mixtures were incubated at room temperature for 30 minutes. The absorbance was measured at 415 nm using micro-plate reader (Versamax Micro plate Reader). Calibration curve was developed by using Quercetin as positive control.

RESULT

Arginase inhibition assay

Eight fractions were subjected to the assay. The result of arginase inhibition assay shown in Table 1.

Antioxidant activity

The result of antioxidant activity assay shown in Table 2. Fraction 3 and 6 are two most active fraction in inhibiting DPPH with IC_{50} 25.98 µg/mL and 48.01 µg/mL respectively.

Determination of total phenolic content

The result of total phenolic content of six fractions from ethyl acetate extract of *Caesalpiniaturtuosa Roxb* are shown in Table. 2. Calibration curve developed using Gallic Acid as standards give maximum absorbance at 600 nm with the equation y = 0.0036x + 0.0416 (r^2 = 0.9988).

Determination of total flavonoid content

The result of total flavonoid content of eight fractions from ethyl acetate extract of *Caesalpiniaturtuosa Roxb* are shown in Table 2. Quercetin was used as standard for developing calibration curve. The measurement gives maximum absorbance at 415 nm with the equation y = 0.0387x – 0.047 (r^2 = 0.9993).

Statistical analysis

The analysis is done to the fraction that has the activity of arginase inhibition to predict the contribution of antioxidant activity, total phenolic content, and total flavonoid content to the inhibition of arginase activity. These analyses were done by applying multiple linear regressions and analysis of correlation using Microsoft Excel 2010. The results are shown in Tables 3-5.

DISCUSSION

Maceration method was chosen to avoid any degradation of thermo labile compounds. Multiple stages maceration was done using hexane, ethyl acetate and methanol subsequently. The ethyl acetate extract was subjected to chromatography column. Gradient polarity of eluent will separate the fraction based on the polarity of the compound. Eight fractions were obtained and numbered following the increase of the polarity.

In, argination of hibition assay, the final concentration of each sample in the well is 100 µg/mL. Basic principal of the arginase inhibition measurement is colorimetric method. Urea Kit contains o-phthalaldehyde, and N-(1-naphthyl)ethylenediamme (NED). Urea was yielded through converting arginine by arginase. So, adding urea to the well could give negative feedback to stop the reaction. Fraction

Table 1: Average arginase inhibition in eight fractions from ethyl acetate extract.

Fraction	arginase inhibition	
	average	SD
1	61.168	4.872
2	63.574	7.241
3	90.722	11.151
4	-9.227	0.809
5	22.264	4.354
6	91.409	0.595
7	49.485	1.458
8	-110.196	7.564

Table 2: Antioxidant activity, total phenolic content and total flavonoid content of six active fractions.

Fraction	Antioxidant activity		Total phenolic content		Total flavonoid content	
	Average	SD	Average	SD	Average	SD
1	14.975	1.527	16.491	0.893	0.081	0.013
2	31.161	1.452	26.259	0.626	0.072	0.003
3	73.664	0.774	56.815	2.98	0.096	0.004
4	68.493	2.815	41.907	0.08	0.13	0.009
5	59.823	1.921	63.667	0.556	0.178	0.007
6	72.414	0.523	114.037	1.165	0.227	0.002
7	49.308	1.127	111.213	0.626	0.212	0.021
8	72.53	0.481	118.481	0.925	0.066	0.002

Table 3: Multiple linear regression output.

Regression Statistics	
Multiple R	0.96
R Square	0.93
Adjusted R Square	0.82
Standard Error	8.11
Observations	6

Table 4: Multiple linear regression output.

	Coefficients	Standard Error	t Stat	P-value	Lower 95%	Upper 95%	Lower 95.0%	Upper 95.0%
Intercept	28.52	11.86	2.40	0.14	-22.52	79.56	-22.52	79.56
antioxidant activity	1.11	0.22	4.99	0.04	0.15	2.07	0.15	2.07
total phenolic content	-0.90	0.30	-2.98	0.10	-2.20	0.40	-2.20	0.40
total flavonoid content	339.47	158.34	2.14	0.16	-341.83	1020.76	-341.82	1020.76

Table 5: Correlation analysis.

	arginase inhibition	antioxidant activity	total phenolic content	total flavonoid content
arginase inhibition	1			
antioxidant activity	0.73	1		
total phenolic content	0.15	0.68	1	
total flavonoid content	0.18	0.56	0.94	1

6 has highest activity, followed by fraction 3. There are two fractions, 4 and 8 have no activity in inhibiting arginase indicated by negative % inhibition.

Antioxidant activity assay, total phenolic determination and total flavonoid determination were conducted to six fractions which have activity on arginase. The aim of these assays was to predict the relationship of these three variables to inhibitory activity. The antioxidant activity assay conducted followed DPPH radical scavenging method. This method was chosen due to simple.[21] The original color of DPPH was purple. Antioxidant will bleach that purple into yellowish through scavenge free radicals by donating electron.

Total phenolic content determination from samples was measured using colorimetric method using Foline-Ciocalteu reagent that will form blue complex with phenolic compound.[1] The darkness of the blue color proportional with the number complex of Folin-Ciocalteu and phenolic compound formed. Total phenolic content was determined by plotting the absorbance of each sample into calibration curve and expressed in milligrams Gallic Acid equivalent (mgGAe). Labelling for the fractions follow the increasing of the polarity of the fractions. Polarity of the fraction could consider based on the composition of the solvent used while column chromatography run. From the result obtained that the level of phenolic compound in the sample increase as the polarity increase. It makes sense because phenolic compounds are relatively polar and polar solvent will solve the similar polarity compound. Combination of ethyl acetate and methanol could attract more polar compound than combination hexane and ethyl acetate could do.

Total flavonoid content was measured based on the number of complex flavonoid with $AlCl_3$ which formed. Total flavonoid content of each sample was obtained by plotting the absorbance into calibration curve and convert to milligrams Quercetin equivalent (mgQE). Differences in total flavonoid content in these eight fractions attribute to the polarity of the fraction. Fraction 6 found had the highest total flavonoid content, followed by fraction 7. As known, more flavonoid is semi polar compound. Thus, flavonoid can easily solve in semi polar solvent like ethyl acetate.

The activity of inhibiting arginase shows moderate relationship with antioxidant activity (r= 0.29), total phenolic content (r= 0.31) and total flavonoid content (r= 0.35). The relationship among arginase inhibition to antioxidant activity and total phenolic content shows negative pattern. These mean increasing the antioxidant activity and total phenolic content are not followed by arginase inhibition activity. It can be considered that arginase inhibitor activity of this plant was not related to antioxidant activity and high phenolic content. Meanwhile, arginase inhibitor activity shows positive relationship with total flavonoid content. However, statistical analysis found no significant relationship between arginase inhibition activity and these three variables.

Since the active compound is still unknown, further study should be done to isolate the constituent that has activity of arginase inhibition from this plant.[22-24]

CONCLUSION

Six fractions form ethyl acetate extract of *Caesalpiniaturtuosa* Roxb found have activity as arginase inhibitor and antioxidant as well. Activity in arginase inhibition of this plant is not related to the total phenolic and total flavonoid content.

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CONFLICT OF INTEREST

There is no conflict of interest.

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